Appropriate control of ex vivo gene therapy delivering basic fibroblast growth factor promotes successful and safe development of collateral vessels in rabbit model of hind limb ischemia

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Purpose: **In our previous study, adenovirus-mediated ex vivo gene transfer of basic fibroblast growth factor promoted significant collateral vessel development in a rabbit model of hind limb ischemia. The present study examined how to control the efficacy and safety of this gene therapy, and also evaluated the feasibility of repeat application of this procedure.**

Methods: **Modified hFGF gene with the secretory signal sequence was adenovirally transferred to cultured autologous fibroblasts, and various numbers of the cells (2×10^5, 1×10^6, 5×10^6, or 2.5×10^7) or vehicle was injected through the left internal iliac artery in rabbits in whom the left femoral artery had been excised 21 days previously. Twenty-eight days after cell administration, calf blood pressure ratio, angiographic score, blood flow in the internal iliac artery, and capillary density of muscle tissue were measured to analyze collateral vessel development and tissue perfusion in the ischemic limb. To assess delivery efficiency and viral contamination, the distribution of injected cells and the time course of blood anti-adenovirus antibody titer were examined in rabbits treated with various numbers of gene-transduced cells. In addition, animals received two injections, 21 days apart, of fibroblasts infected with adenovirus vector containing the luciferase gene, and luciferase expression was measured to evaluate whether the present therapy is repeatable.**

Results: **At 28 days after cell administration, significant collateral vessel development without detectable side effects was observed in rabbits who received 5×10^6 or 2.5×10^7 cells, compared with those who received vehicle, and no significant development was detected in animals with fewer than 5×10^6 cells ($P < .01$ for calf blood pressure ratio and capillary density, $P < .05$ for angiographic score and maximum blood flow). There was no difference in collateral augmentation between rabbits with 5×10^6 and 2.5×10^7 cells. However, in animals with 2.5×10^7 cells a large number of injected cells accumulated in the lungs, anti-adenovirus antibody titer increased significantly, and calf blood pressure in the left hind limb of two rabbits decreased immediately after injection. Luciferase analysis showed very low gene expression after repeated administration.**

Conclusion: **These findings suggest that 5×10^6 is a suitable number of cells to induce appropriate collateral vessel development and minimize potential side effects of this procedure. Despite use of ex vivo gene transfer, repeat administration of the cells was not feasible. (J Vasc Surg 2004;39:629-38.)**

Clinical Relevance: **Since the present study determined the appropriate conditions for effective and safe stimulation of collateral vessels, the clinical relevance of the ex vivo therapy might be carried forward. However, the findings raised another issue that should be resolved before clinical application; that is, the number of gene-transduced cells able to be injected was strictly limited. To estimate the therapeutic range of cell number in humans, additional experiments using large animals are desirable.**

To enhance collateral vessel development for treatment of vascular occlusive disease, a variety of techniques have been presented for local delivery of angiogenic growth factor, such as basic fibroblast growth factor (bFGF) or vascular endothelial growth factor, among others. Direct administration of recombinant protein to deliver these growth factors was initially investigated,[1,2] and subsequently several protocols for gene transfer were established to realize more efficient and sophisticated delivery.[3-5] In previous studies we presented an alternative strategy using ex vivo gene transfer of bFGF, and demonstrated significant augmentation of collateral vessel development and tissue perfusion in a rabbit model of hind limb ischemia.[6] The concept of this strategy was that autologous fibroblasts, transduced with modified bFGF gene, were selectively injected into the donor artery. The donor artery indicates a potential origin of a collateral vessel network

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Supported by a Grant-in Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (13557098).

Competition of interest: none.

Additional material for this article may be found online at www.mosby.com/jvs.

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0741-5214/$30.00

doi:10.1016/j.jvs.2003.09.016

toward the ischemic lesion, and requires adequate blood inflow for blood supply. The reason for using fibroblasts as the delivery carrier was that the fibroblast is a non–blood cell that is readily feasible for primary culture. On this account, the gene-transduced fibroblasts were physically trapped in peripheral small vessels of the donor artery, and secreted bFGF for a certain period, because the modified bFGF gene was fused with the secretory signal sequence of interleukin-2.[7,8] Angiogenesis and arteriogenesis commonly occur in small arteries and capillaries. This strategy therefore achieves specific delivery of bFGF to the ideal target to promote favorable collateral vessel development with sufficient blood inflow. Further, with this strategy the bFGF gene was transferred into fibroblasts with an adenovirus vector, because of its high efficiency of gene transfer[9] and transient expression of the transduced gene.[10,11] Transient gene expression would be a favorable feature for safety, because long-term expression of growth factor gene would increase the possibility of unexpected side effects.

Our therapeutic strategy has some unique features in inducing effective development of collateral vessels. However, some issues must be resolved before clinical application. Most important is how to control the effect and safety of this therapy. Inasmuch as the bFGF gene was transferred to all fibroblasts before administration, a variable factor that influences performance of this therapy is the number of administered fibroblasts. Our previous study only showed data after administration of 5×10^6 gene-transduced fibroblasts. Further, there is little evidence of how non–blood cells, in which an exogenous gene is transduced, function and behave after administration into a peripheral artery. In the present study we therefore determined a suitable cell number to promote appropriate collateral vessel development and minimize the possibility of side effects. In addition, we focused on repeat application of the ex vivo procedure. Because our previous study reported no significant induction of humoral immune response against adenovirus after injection of gene-transduced cells,[6] we considered the possibility that ex vivo therapy with an adenovirus vector could be repeated without early elimination of the administered cells.

METHODS

Adenovirus. Replication-deficient recombinant adenovirus vector containing modified human bFGF gene with the secretory signal sequence of interleukin-2 (AxCAMAssbFGF) was constructed with a method utilizing cosmid cassettes and adenovirus DNA-terminal protein complex (the COS-TPC method).[12,13] Modified human bFGF is more stable than the original human bFGF,[8] and synthesized bFGF can be secreted to the outside of cells, because of the added secretory signal sequence. Our previous study showed that rabbit fibroblasts infected with AxCAMAssbFGF significantly secreted bFGF into the culture medium.[6] AxCALacZ containing the *Escherichia coli* LacZ gene in the same expression unit was used as a control vector for evaluation of side effects, and AxCAluc+ containing recombinant luciferase gene (luc+) was used to evaluate in vivo expression of the transduced gene.

Animal model of chronic hind limb ischemia. For evaluation of angiogenic responses in vivo, we used a rabbit model of hind limb ischemia.[6,14] Male Japanese White rabbits weighing 3.0 to 3.5 kg (Saitama Rabbitry, Saitama, Japan) were anesthetized, and the left femoral artery was completely excised, from its proximal origin to the bifurcation formed by the saphenous and popliteal arteries.[14] Before closure of the wound, a 10×20 mm section of skin was obtained for primary culture of fibroblasts (Fig 1). All protocols were in accordance with the Guide for Animal Experimentation of The University of Tokyo.

Ex vivo gene transfer. Fibroblasts were cultured from the resected skin tissue and grown to confluence in 100-mm dishes, as described.[6] At 20 days after femoral artery excision the fibroblasts (passage 3) were infected in vitro with AxCAMAssbFGF at 20 plaque-forming units (pfu) per cell and incubated for 24 hours.[15] At 21 days after removal of the femoral artery, a 3F end-hole infusion catheter (Rapid Transit; Cordis, Miami, Fla) was introduced into the carotid artery and positioned in the left internal iliac artery. After washing three times with phosphate-buffered saline solution, the infected fibroblasts were suspended in 4.5 mL of Dulbecco modified Eagle minimum essential medium (DMEM; Gibco BRL, Grand Island, NY) with 2% fetal bovine serum (DMEM 2%), then injected through the infusion catheter (Fig 1). In this study various numbers of gene-transduced fibroblasts were administered in four groups of six rabbits each, as follows: 2×10^5, 1×10^6, 5×10^6, and 2.5×10^7. Another group of seven rabbits was injected with 4.5 mL of DMEM 2% (vehicle) as control.

Assessment of collateral vessel development and tissue perfusion. Calf blood pressure (BP) in both hind limbs was measured with a 2.5-cm wide cuff and Doppler scanning probe, and the calf BP ratio was calculated as the ratio of left systolic pressure to right systolic pressure.[2] Calf BP ratio was assessed four times: immediately after femoral artery excision, and immediately before, immediately after, and 28 days after administration of infected cells or vehicle. At 28 days after injection of gene-transduced fibroblasts or vehicle, selective internal iliac arteriography was performed, and angiographic score was calculated, as described.[5] Before angiography, resting blood flow in the left internal iliac artery and maximum blood flow after papaverine injection were measured with a Doppler guide wire, as described.[16] After the animals were killed, tissue specimens were obtained as transverse sections from the left semimembranous muscle. Frozen sections were cut (two sections per animal), capillary endothelial cells were stained with the indolyltetrazolium method,[17] and capillaries were counted in 20 different fields from the two sections to determine capillary density.[2]

Distribution of administered cells. To evaluate the distribution of injected cells, cultured fibroblasts were radiolabeled with indium 111–oxine solution (Amersham Pharmacia Biotech, Little Chalfont, England) according to

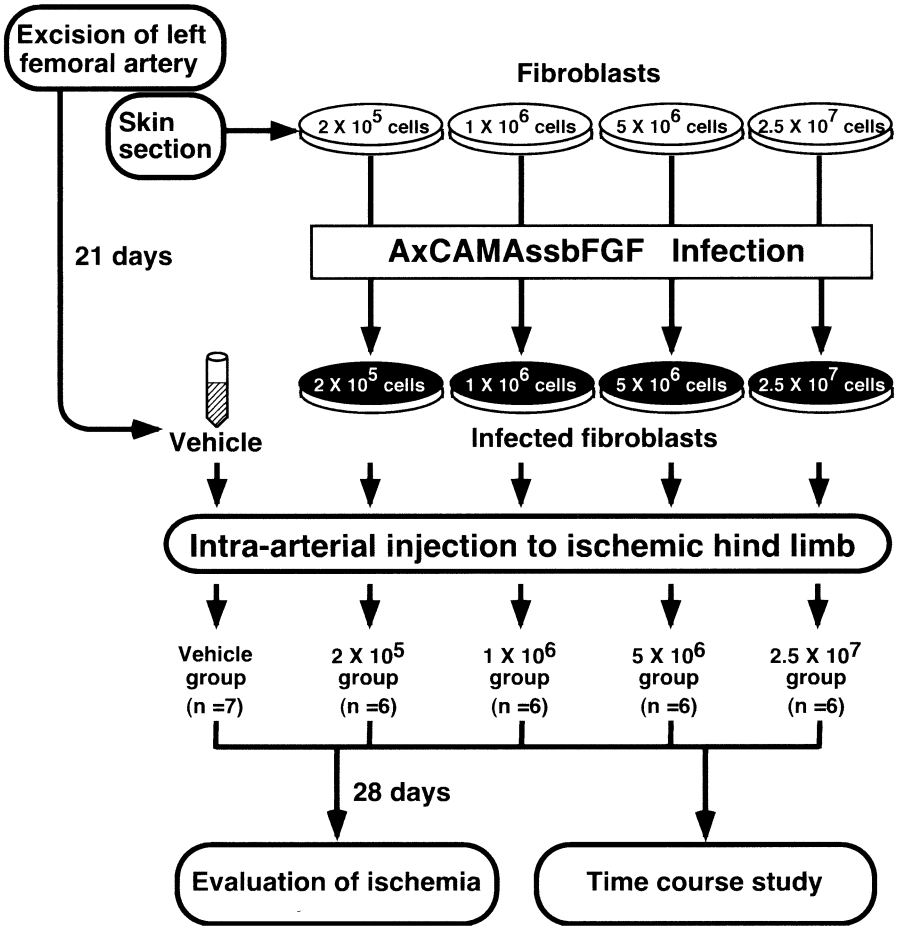

Fig 1. Experimental protocol for intra-arterial injection of various numbers of gene-transduced fibroblasts.

the protocol described.[6] At 21 days after removal of the left femoral artery the labeled fibroblasts suspended in 4.5 mL of DMEM 2% were injected through the left internal iliac artery. We tested three cell numbers in this study: 1×10^6 ($n = 5$), 5×10^6 ($n = 7$), and 2.5×10^7 ($n = 5$). Treated animals were killed 5 hours after cell administration, and above-knee muscles, below-knee muscles, and skin were taken from the bilateral hind limbs, and internal organs were collected. The distribution (percentage) of cells in each muscle or organ was determined as described.[6] In addition, the cell number distributed to each muscle or organ was calculated as (Distribution %)×(Administered cell number)/100.

In vivo expression of bFGF and side effects. At 21 days after removal of the left femoral artery, 5×10^6 or 2.5×10^7 fibroblasts treated with AxCAMAssbFGF were injected through the left internal iliac artery. For time course study the rabbits were killed, at 7, 14, 21, and 28 days after injection ($n = 5$ at each time point), and various samples were taken from the bilateral adductor muscles, lung, heart, liver, kidney, spleen, testis, serum, and total blood. Some rabbits ($n = 5$ at each time point) were injected with 4.5 mL of DMEM 2% (vehicle), and another group of animals ($n = 5$) was killed at 21 days after femoral artery excision as control (control). To determine in vivo bFGF expression, the left adductor muscle (1 g), lung (0.5 g), and liver (0.25 g) were analyzed with the Western blot technique, after concentration with heparin-sepharose CL-6B (Amersham Pharmacia Biotech), as described.[18] The primary antibody used in the Western blot technique recognizes endogenous rabbit bFGF and also the bFGF synthesized by AxCAMAssbFGF infection. Serum bFGF concentration was quantified with an enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, Minn), and anti-adenovirus antibody titer with a neutralization test (SRL, Tokyo, Japan).[6] Side effects were assessed with blood examination and histologic evaluation. On the time course blood samples we performed a complete blood cell count and blood chemical tests (SRL), including aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, total bilirubin, alkaline phosphatase, urea nitrogen, and creatinine concentration. Further, each tissue sample was embedded in paraffin, and sections were stained with hematoxylin-eosin (HE) or elastica–van Gieson (EVG) and analyzed.

For evaluation of the inflammatory reaction in host tissue, frozen sections of the left adductor muscle were immunostained with monoclonal antibody against rabbit macrophage (RAM-11, 1:50; DAKO, Copenhagen, Denmark), CD18+ leukocyte (L13/64, 1:100; Serotec, Oxford, England), or T lymphocyte (L11/135, 1:100; Serotec), as described.[19,20]

Administration of gene-transduced cells to venous system. To assess the side effects of gene-transduced cells that entered the venous system, AxCAMAssbFGF-treated fibroblasts (1×10^7) were injected through the iliac vein of normal rabbits, and the influence on the lung was analyzed. Blood gas analysis was carried out at 1, 4, 7, 14, 21, and 28 days after intravenous injection of infected cells. Lung samples were obtained at days 7 and 28, and were examined histologically (HE, EVG). Control animals were treated with AxCALacZ.

Repeated administration of gene-transduced fibroblasts. To evaluate whether the present ex vivo therapy is repeatable, AxCAluc+-treated fibroblasts were administered twice, at an interval of 21 days. First, 5×10^6 fibroblasts, infected with AxCAluc+, were injected through the left internal iliac artery 21 days after removal of the left femoral artery (first challenge). Twenty-one days later AxCAluc+-treated fibroblasts (5×10^6) were administered to the same rabbits in the same manner (second challenge), and the animals were killed on day 7, 14, 21, or 28 after the second challenge (n = 5 at each time point) for sampling the left above-knee muscles (repeat group). Another group of rabbits (n = 5 at each time point) was injected with AxCAluc+-treated fibroblasts (5×10^6) 21 days after administration of noninfected fibroblasts as control (control group). Each sample was weighed (W_{Whole}), minced, and homogenized. A certain volume of the homogenized sample (approximately 0.5 g, W_{Sample}) was lysed in 1 mL of luciferase cell culture lysis buffer (Promega, Madison, Wis), and the volume of the lysate was measured (V_{Lysate}). After 100 μL of luciferase substrate (Promega) was added to 20 μL of each lysate, luciferase activity was measured with a luminometer (Lumat LB9507; Berthold Technologies, Bad Wildbad, Germany), and luciferase concentration (C_{Sample}) was calculated from the luciferase activity with a standard curve made by purified firefly luciferase (Promega).[21] Luciferase content of above-knee muscles was calculated as: $C_{Sample} \times (W_{Whole}/W_{Sample}) \times (V_{Lysate}/20\mu L)$.

Statistical analysis. Counting for determination of angiographic score and capillary density was performed by a single student helper in a blind fashion. Results are expressed as mean ± SD. The Tukey-Kramer method was used for analysis of collateral vessel development and tissue perfusion. Student's *t* test was used for analysis of the distribution of administered cells. Repeated-measures analysis of variance was used for analysis of systemic bFGF concentration, after subtracting preinjection data from the data at each time point. Repeated-measures analysis of variance, after logarithmic transformation followed by a simple main effects test, was used for analysis of antiadenovirus titer. All data were considered significant at $P < .05$. All statistical analyses were performed with SAS version 8.2 (SAS Institute, Cary, NC).

RESULTS

Assessment of collateral vessel development and tissue perfusion. Calf BP ratio showed no significant difference between all groups before administration of cells or vehicle. At 28 days after injection calf BP ratio in the 5×10^6 and 2.5×10^7 groups was significantly higher than in the other three groups, whereas no significant difference was detected between the 5×10^6 and 2.5×10^7 groups or between the other three groups. Meanwhile, calf BP decreased to zero only in two rabbits in the 2.5×10^7 group when measured immediately after injection to evaluate the influence of intra-arterial injection of fibroblasts (Fig 2). Angiograms obtained 28 days after injection showed well-developed collateral vessels in the 5×10^6 and 2.5×10^7 groups compared with the 2×10^5, 1×10^6, and vehicle groups (Fig 3, *a-e*), which coincided with the angiographic score (Fig 3, *f*). Capillary density in the 5×10^6 and 2.5×10^7 groups was significantly higher than in the other three groups at 28 days after injection (Fig 4, *a*). Furthermore, in the 5×10^6 and 2.5×10^7 groups, blood flow in the left internal iliac artery at rest was significantly higher than in the vehicle group (Fig 4, *b*), and maximum blood flow was significantly greater than in the other three groups (Fig 4, *c*).

Distribution of administered cells. Distribution data (%) showed that about 45% of labeled cells accumulated in the above-knee and below-knee muscles of the left hind limb in animals treated with 1×10^6 or 5×10^6 cells (Fig 5, *a* and *b*). Only in rabbits treated with 2.5×10^7 cells, great accumulation was observed in the lungs, but only 10% of labeled cells accumulated in the above-knee and below-knee muscles (Fig 5, *c*). Although the distribution (%) in the above-knee and below-knee muscles of the left hind limbs differed according to the number of injected cells, there was no significant difference in accumulated cell number between animals treated with 5×10^6 and 2.5×10^7 cells (Fig 5, *d*).

In vivo expression of bFGF protein. The Western blot technique showed local accumulation of expressed bFGF. In rabbits treated with 5×10^6 or 2.5×10^7 cells, bFGF accumulation in the adductor muscle was increased 7 and 14 days after cell administration, compared with that in control and vehicle-treated animals (Fig 6, *a* and *b*). bFGF accumulation decreased thereafter, although the amount of bFGF on days 21 and 28 was slightly higher than that in vehicle-treated rabbits. At 7 days after cell injection bFGF accumulation in the adductor muscle was increased in rabbits treated with 2.5×10^7 cells, compared with those treated with 5×10^6 cells, but no distinct difference was detected between the two groups on days 14, 21, and 28. In the lung and liver the time course of bFGF level revealed no remarkable difference from that in control animals (Fig 6, *c* and *d*).

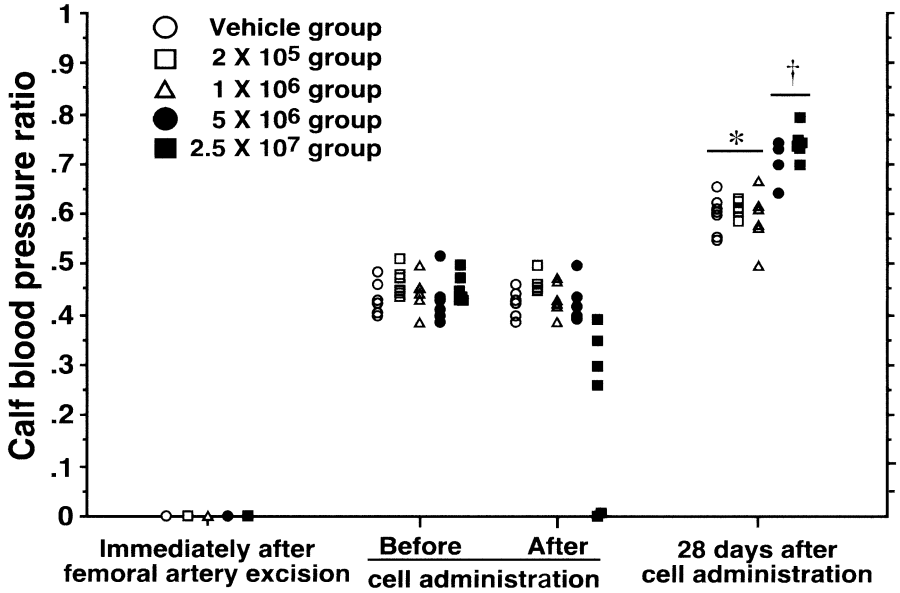

Fig 2. Calf blood pressure ratio immediately after femoral artery excision, and immediately before, immediately after, and 28 days after cell administration. At 28 days there were significant differences between each group in line * and each group in line † ($P < .01$). There were no significant differences among the groups within each line.

Systemic bFGF level and anti-adenovirus antibody titer. Time course analysis of systemic bFGF level showed no significant increase in bFGF in rabbits administered 5×10^6 or 2.5×10^7 cells or vehicle, and there was no significant difference between the three groups (Fig 7, *a*). Anti-adenovirus titer in animals injected with 2.5×10^7 cells was significantly higher than that in animals with 5×10^6 cells on days 14, 21, and 28 (Fig 7, *b*).

Side effects and inflammatory reactions. Neither complete blood cell count nor blood chemical tests showed any abnormal data in animals treated with 5×10^6 or 2.5×10^7 cells at any time point after cell injection (data not shown), and histologic studies with HE and EVG staining revealed no fibrosis, infarction, degeneration, or other abnormal changes in any tissues up to day 28 (data not shown). Inflammatory reaction in the left adductor muscle was evaluated with immunostaining for macrophages, CD18+ leukocytes, or T lymphocytes, and no remarkable infiltration of these cells was observed at 7 days after injection, as compared with vehicle-treated samples (Fig 8, online only).

Intravenous injection of gene-transduced fibroblasts. Blood gas analysis showed no significant changes in Pao_2, $Paco_2$, or other parameters throughout the time course after injection of AxCAMAssbFGF-treated cells, and no difference compared with AxCALacZ-treated control (Fig 9, *a* and *b*, online only). In addition, histologic studies showed no abnormal findings, such as fibrosis, compared with control at 7 and 28 days after injection (Fig 9, *c* and *d*, online only).

Repeated administration of gene-transduced fibroblasts. In the control group, marked increase of luciferase expression was observed in the left above-knee muscles, which was maximal 7 days after the second challenge and decreased thereafter (Fig 10). In contrast, in the repeat group, which received injection of AxCAluc+-treated cells twice, very low level expression of luciferase was detected throughout the time course (Fig 10).

DISCUSSION

We administered various numbers of AxCAMAssbFGF-treated fibroblasts into the rabbit ischemic hind limb through an intra-arterial catheter, and several findings suggest that injection of 5×10^6 fibroblasts is a suitable amount to induce appropriate development of collateral vessels and possibly prevent side effects in a rabbit model of chronic hind limb ischemia.

First, a certain cell number was required for significant induction of collateral vessels (minimum requirement). Despite no significant increase of collateral vessel development in the 2×10^5 and 1×10^6 groups, well-developed collateral vessels were quickly detected in the animal groups treated with more than 5×10^6 cells. That is, if the number of injected fibroblasts was less than the minimum requirement, which was between 1×10^6 and 5×10^6, administration of gene-transduced cells promoted no meaningful effect. The volume of ischemic tissue must be one critical factor that determines minimum requirement. In addition, the cells' ability to release bFGF might also be important. The conditions of adenovirus infection, such as virus concentration, possibly influence the ability of bFGF release. We further speculate that the level of ischemia of the tissue is another important factor that determines minimum requirement, because our previous study showed that collateral vessel development in cell-administered tissues depended on the level of ischemia in the tissues.[6] Of interest,

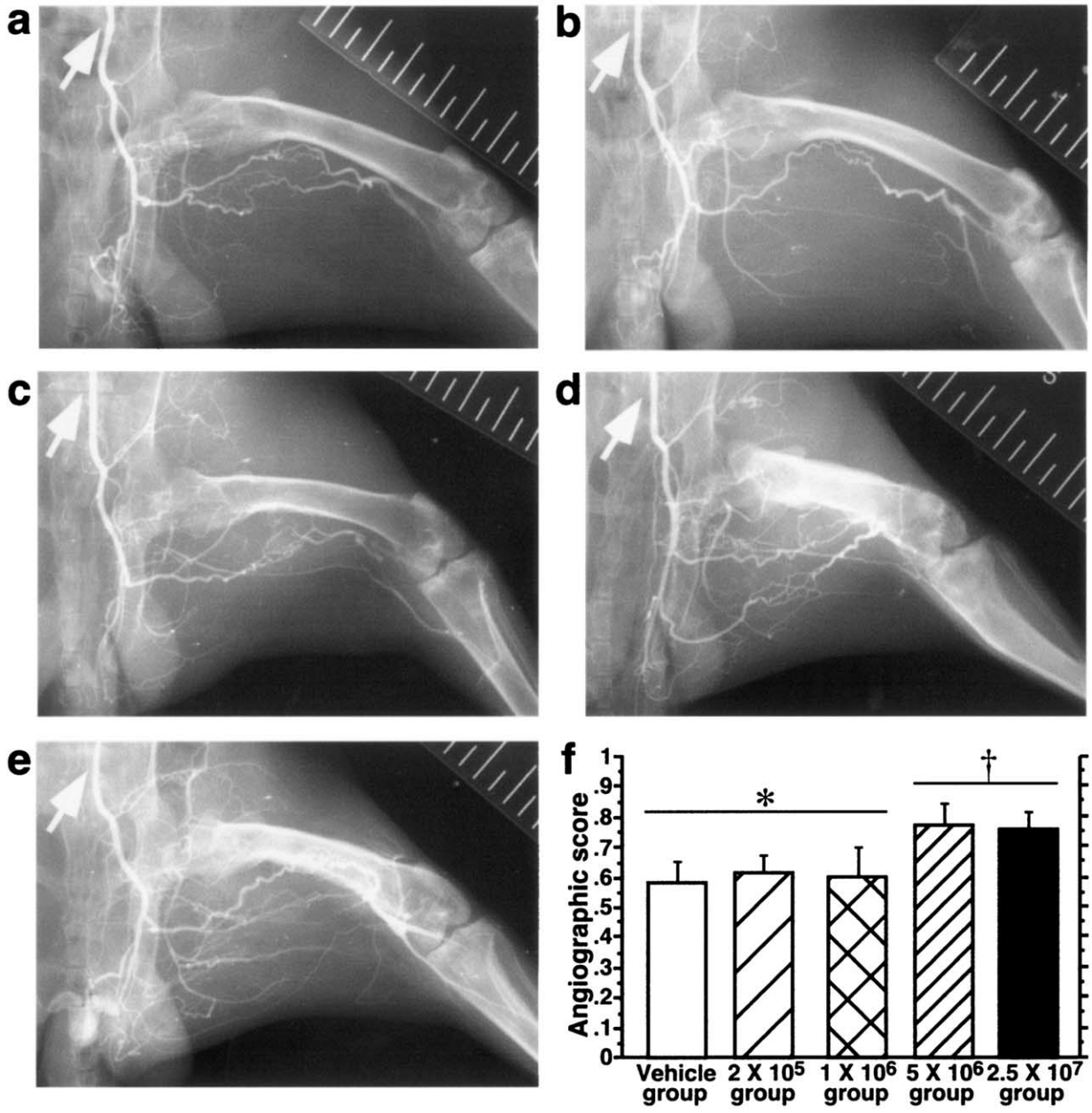

Fig 3. Selective internal iliac angiograms of rabbits at 28 days after injection of vehicle **(a)** and 2×10^5 **(b)**, 1×10^6 **(c)**, 5×10^6 **(e)**, and 2.5×10^7 **(e)** AxCAMAssbFGF-tranduced fibroblasts. *Arrow,* internal iliac artery. **f,** Development of collateral vessels was quantified by angiographic score. There were significant differences between each group in line * and each group in line † ($P < .05$). There were no significant differences among groups within each line.

two study groups[1,22] reported a similar dose-response pattern in their studies with naked DNA of acidic FGF gene or bFGF protein.

Second, in considering the suitable cell number, administration of larger numbers of cells did not induce further development of collateral vessels above a certain level. Indeed, all parameters of collateral vessel augmentation in the present study revealed no significant difference between the 5×10^6 and 2.5×10^7 groups. One possible explanation is the capacity of the limb muscles to retain the cells in their capillaries and small arteries. At 5 hours after injection of labeled fibroblasts, the number of accumulated cells in the left hind limb muscles showed no significant difference between animals treated with 5×10^6 and 2.5×10^7 cells, whereas the cell distribution (%) revealed greater cell accumulation in the lungs of rabbits treated with $2.5 \times$

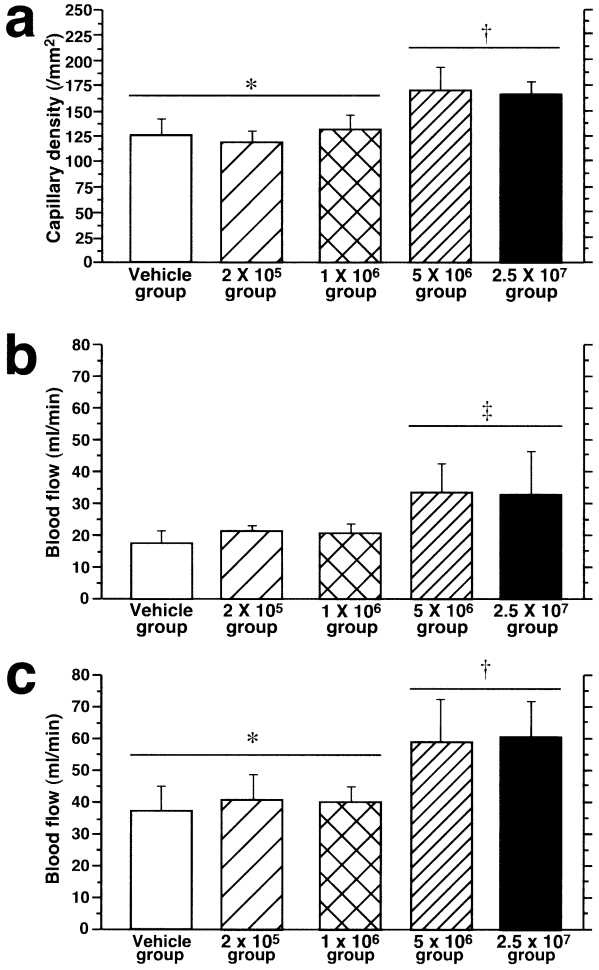

Fig 4. a, Capillary density of left semimembranous muscle. **b,** In vivo blood flow of left internal iliac artery at rest. **c,** Maximum in vivo blood flow of left internal iliac artery. There were significant differences between each group in line * and each group in line † ($P < .01$ for capillary density; $P < .05$ for maximum blood flow). There were no significant differences between groups within each line. For blood flow at rest **(b)** there were significant differences between each group in line ‡ and the vehicle group ($P < .05$), but no significant differences among the groups within this line.

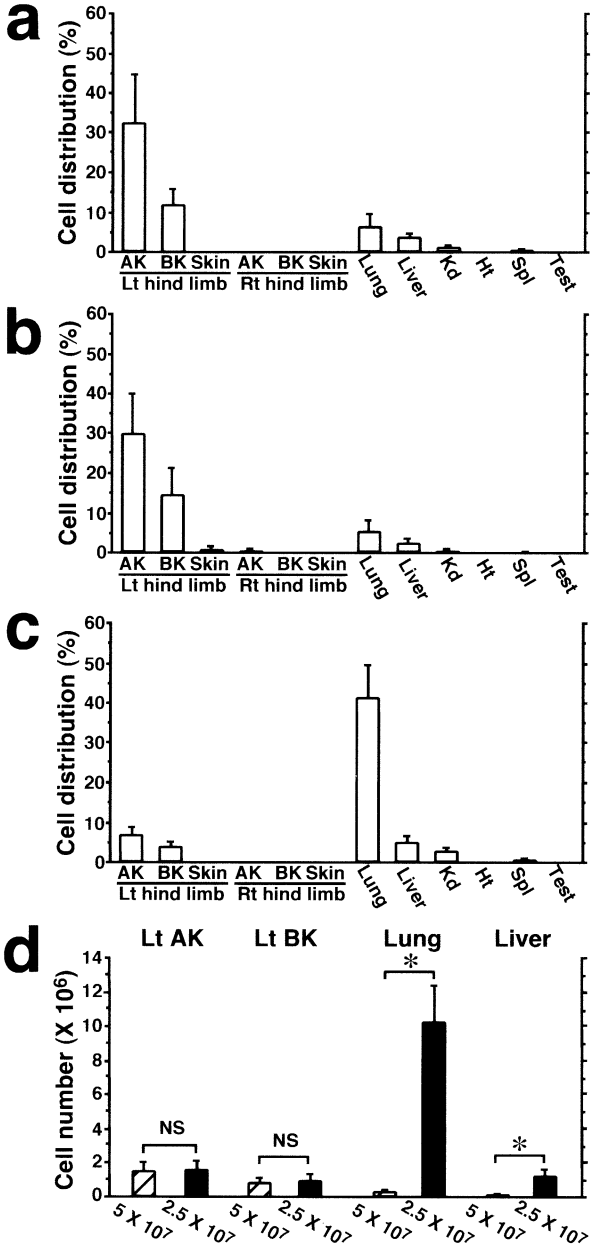

Fig 5. Distribution of indium 111–labeled fibroblasts in organs and tissues at 5 hours after injection of 1×10^6 cells **(a)**, 5×10^6 cells **(b)**, and 2.5×10^7 cells **(c)** into the left internal iliac artery. Data are presented as percentage of radioactivity distributed in each tissue relative to total radioactivity of administered cells. **d,** Cell number of ^{111}In-labeled fibroblasts in above-knee and below-knee muscles of left hind limb, lung, and liver at 5 hours after injection of 1×10^6, 5×10^6, and 2.5×10^7 cells into the left internal iliac artery. *$P < .001$. *AK,* Above-knee muscles; *BK,* below-knee muscles; *Lt,* left; *Rt,* right; *Kd,*; kidney; *Ht,* heart; *Spl,* spleen; *Test,* testis; *NS,* no significant difference.

10^7 cells. These findings suggest that surplus cells exceeding the capacity of the tissue overflowed into the venous system and were trapped in the lungs, increasing the possibility of unexpected pulmonary side effects. Results of the Western blot technique on the left adductor muscle samples showed no notable difference in bFGF expression between rabbits treated with 5×10^6 and 2.5×10^7 cells, supporting the above concept.

Another important finding was that calf BP ratio decreased to zero immediately after injection in two rabbits in the group treated with 2.5×10^7 cells, which indicated worsening of ischemia. However, no significant decrease in calf BP ratio was detected after injection of 5×10^6 or fewer cells. These findings indicate that the excessive fibroblasts behaved like emboli in the capillaries and small arteries, and reduced peripheral blood flow immediately after injection,

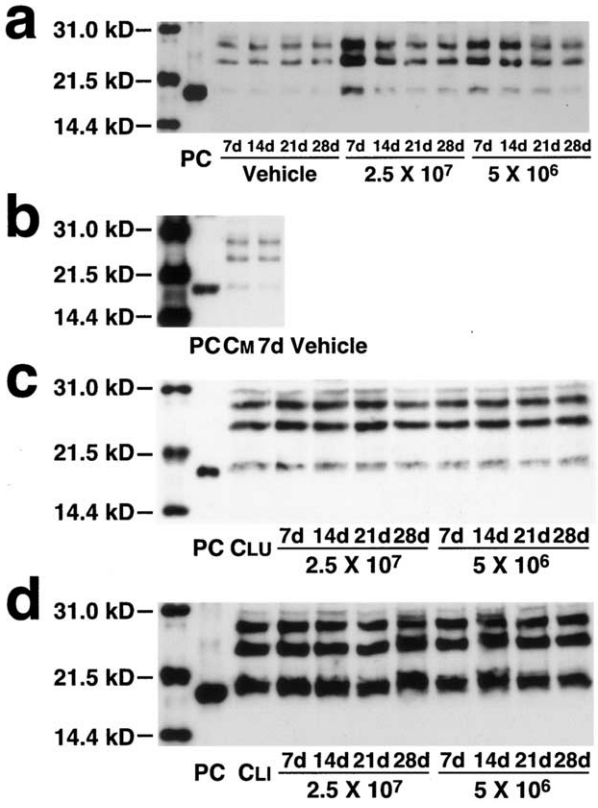

Fig 6. bFGF accumulation in left adductor muscle **(a, b)**, lung **(c)**, and liver **(d)** with Western blot technique. **b,** bFGF accumulation of control animals (21 days after femoral artery excision) was similar to that in vehicle-treated animals. *PC*, Positive control; *vehicle,* vehicle-treated sample; *CM*, left adductor muscle sample from control animal; *CLU*, lung sample from control animal; *CLI*, liver sample from control animal; *d,* days after infection.

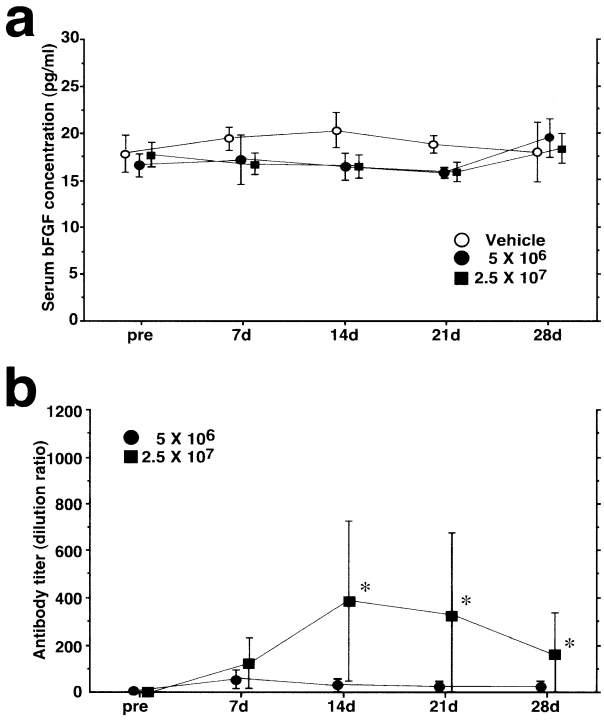

Fig 7. a, Time course of systemic bFGF level measured with ELISA. **b,** Time course of anti-adenovirus antibody titer quantified with neutralizing test. Titers are shown as dilution ratio, and titers less than 1:4 were assigned a value of 1. * $P < .0001$ (5×10^6 cells vs 2.5×10^7 cells). *pre,* Preinjection; *d,* days after injection.

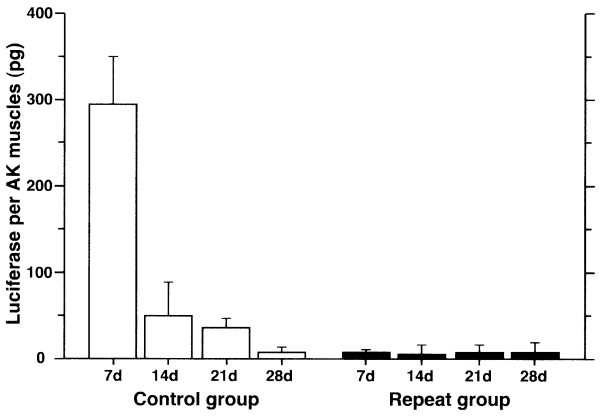

Fig 10. Expression of luciferase in left above-knee *(AK)* muscles of rabbits. *d,* Days after second challenge.

which is possibly critical in ischemic tissue. Serum anti-adenovirus antibody level in rabbits treated with 2.5×10^7 cells was significantly higher than that in animals treated with 5×10^6 cells, indicating that the host was contaminated with viral particles. This might be because three washes after viral infection was insufficient to remove viral particles from 2.5×10^7 cells. Because the adenovirus vector used in this procedure is replication-deficient, contamination with viral vector may not induce severe side effects; however, attention should be paid to the appearance of replication-competent viruses.

To apply the study protocol to the clinical setting, it is necessary to assess safety in other nonischemic tissues and organs. Although the maximum number of injected cells was five times that in our previous study, no detectable change was observed in the evaluations performed. One possible reason is that there was no significant increase in systemic bFGF level over the course of the study. If secreted bFGF completely entered the bloodstream, 5×10^6 cells would be enough to significantly elevate the systemic bFGF level, because in vitro analysis in the previous study showed that rabbit fibroblasts treated in the same manner secreted 25 pg of bFGF per 1000 cells per 24 hours into the culture medium.[6] The binding ability between bFGF and proteoglycans of the extracellular matrix[23] might function to maintain bFGF in the targeted tissues and contribute to continuing the effects of bFGF. In our previous study a slightly increased amount of bFGF was detected in the left adductor muscle until 28 days after cell injection, although

immunohistologic analysis revealed that the number of bFGF-positive cells in the sample muscle sample returned to the control level by day 14.[6] Another possible explanation for the negative findings in the safety assessment is that the number of gene-transduced cells was too low to induce any detectable abnormality in the evaluations in the present study. Especially in the lungs of the animals treated with 2.5×10^7 cells, the Western blot technique revealed no detectable increase in bFGF, although approximately 1×10^7 cells were distributed to the lungs in these animals. We speculate that even the amount of bFGF secreted by 1×10^7 cells might be minimal, compared with that of bFGF that the entire bilateral lung tissue contained. Indeed, in our previous study immunohistologic analysis showed that no significant change in the number of bFGF-positive cells was detected in the lungs.[6] Intravenous injection of 1×10^7 AxCAMAssbFGF-treated fibroblasts also produced no detectable changes in blood gas analysis or histologic findings, which supports these results. Thus the number of cells used in this protocol might be within safety limits.

A disappointing result of the present study is that repeated application of this ex vivo gene therapy did not appear to provide additional efficacy. In several experiments with animal models administration of adenovirus vector induced humoral and cellular immune responses against adenovirus, which possibly limited expression of the transduced gene. Contrarily in the present study, few particles of the adenovirus vector were directly administered into the host artery, as a result of ex vivo gene transfer. Indeed, in both of our studies titers of serum anti-adenovirus antibody remained at less than 1:100 up to 28 days after administration of 5×10^6 cells,[6] and immunohistologic analysis revealed no notable inflammatory infiltration in the left thigh muscles. Further, a recent study reported that repeated treatment with adenovirus vector was feasible in mice with anti-adenovirus antibody titer of 1:600.[24] We therefore hypothesize that the gene-transduced cells of the second challenge could escape early elimination through humoral immune responses, then function for a certain period. However, luciferase analysis in the present study showed very low gene expression even at 7 days after repeated administration of AxCAluc+-treated cells, which contradicts this hypothesis. There is a possibility that neutralizing antibody at a titer of less than 1:100 eliminates the gene-transduced fibroblasts, although a more probable mechanism for the early decrease in gene expression might be cytotoxic immune reaction of T cells. Yang et al[25] found that in a mouse lung model cellular immune responses after the second adenovirus challenge occurred earlier than those after the first challenge. To prolong the life of repeatedly administered cells, it might therefore be necessary to suppress host immune responses with an immunosuppressive drug, but immunosuppression possibly would inhibit development of collateral vessels, because the inflammatory reaction around small arteries induces arteriogenesis.[26] Thus we consider that application of a newer generation of adenovirus vector is more practical than repeated treatment to prolong the duration of gene expression with the present strategy. However, if a newer generation of adenovirus prolongs bFGF expression, it might be necessary to assess the positive and negative influences of long-term delivery of human gene-derived bFGF.

Finally, we must address some other issues that the present study did not resolve. One is the influence of this ex vivo therapy at later times. Our data were limited to 28 days after cell administration. In our previous study[6] gene-transduced cells were eliminated by 14 days after cell injection, although there is the possibility that chronic side effects, such as oncogenesis and worsening of diabetic retinopathy, could develop after day 28. A more important issue is how to apply this protocol to clinical testing. In this ex vivo therapy there is a strict therapeutic range of cell numbers for administration, and injection of excess gene-transduced cells possibly induces critical worsening of tissue ischemia. However, it might be difficult to predict the therapeutic range in human beings from the present data, because there is large species difference between human beings and rabbits. Thus additional experiments with larger animals, such as nonhuman primates, are needed to estimate the therapeutic range in human beings.

In conclusion, this study demonstrated that 5×10^6 gene-transduced fibroblasts is an appropriate number to administer through the internal iliac artery in a rabbit model of hind limb ischemia to promote appropriate development of collateral vessels and minimize side effects. In addition, this ex vivo gene transfer could not be repeated. Since the present ex vivo therapy has a variety of advantageous properties, we believe that suitable administration would induce effective and safe development of collateral vessels in lesions in human beings.

We thank Dr Junji Kishimoto (Department of Clinical Bioinfomatics, The University of Tokyo) and Dr Yasuo Ohashi (Department of Biostatistics, The University of Tokyo) for assistance with the statistical analysis.

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Submitted May 4, 2003; accepted Sep 3, 20003.

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